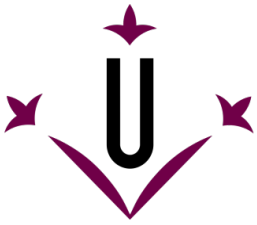

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Substrate-borne vibrational signals in mating communication of Macrolophus bugs

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Response to Reviewers:	We attach a file entitled "response to reviewers" that has the comments of the reviewers and our responses to each of them. We have made minor changes in the manuscript structure: 1-The introduction has been reduced by about 40% 2-A new supplementary figure (S1) has been added

	All the comments of the reviewers have been addressed and for the most part we agree with them and made the oportune changes in the manuscript

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Substrate-borne vibrational signals in mating communication of *Macrolophus* bugs

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Abstract The mirid bugs *Macrolophus pygmaeus* and *M. costalis* use substrate-borne vibrational signals during pair formation and in male-male interactions as determined by laser vibrometry. The vibrational communication of *Macrolophus* is more complex than in other mirids, with a signal repertoire composed of two elements, only produced by males, while the females are mute. The "yelp" signal consists of one or several consecutive brief pulses with ~~modulated~~ harmonic structure and is commonly produced by stationary males before mating, as a key-element of courtship. "Yelping" is also associated with contacts between males. The "roar" signal differs from "yelps" in that it has a broadband frequency pattern, a longer and more variable duration than "yelping", and is produced by males in association with walking on the leaf. Playback experiments did not affect male ~~sound~~ vibration emission, but when "roaring" was used as stimulus, it elicited a significant increase in the time spent walking. We detected significant differences between *M. costalis* and *M. pygmaeus* in some spectral parameters of the "roar" and "yelp" signals, so these signals could contain species-specific information. We conclude that "roaring" and "yelping" vibrational signals are used by *Macrolophus* in social communication, in particular in the context of mating behavior.

Keywords Substrate borne communication, miridae, courtship

Introduction

The omnivorous mirid bug *Macrolophus pygmaeus* (Rambur) (previously *M. caliginosus*, Castañée et al. 2013), is an efficient predator of several key pests in Eeuropean vegetable crops (Alomar et al. 2006; Perdikis et al. 2011; Zappalà et al. 2013; Péerez-Hedo and Urbaneja 2014). ~~Wild host plants maintain predator populations in the landscape and therefore contribute to the colonization of newly planted crops. (Alomar et al. 2002; Castañé et al. 2004; Gabarra et al. 2004; Lykouressis et al. 2008).~~ The mating behavior of *M. pygmaeus* has been described previously, but with no mention about vibrational communication (Castañée et al. 2007; Gemeno et al. 2007). Males walk actively on the plant and upon contact with a female they immediately attempt copulation, which lasts for about 5 min (Gemeno et al. 2007). ~~Mating takes place throughout the 24 h day period, but it is more frequent during the night and first half of the day (Gemeno et al. 2007).~~ A single mating is enough to fertilize most of the ova, and once mated~~,~~ females will not accept a second mating, and will not become receptive even after two weeks ~~later~~, which makes *M. pygmaeus* one of the few monandrous mirid species described (Gemeno et al. 2007; Franco et al. 2011). ~~After repeated male mounting attempts most mated females will leave the plant. Males on the other hand, will remate within minutes after having mated and can do it several times in a row (Gemeno et al. 2007).~~

~~In a monandrous and polygynous species, like *M. pygmaeus*, receptive females will disappear rapidly from the mating pool and the operational sex ratio will quickly move towards an excess of ready-to-mate males and unreceptive females. Under these conditions male-male competition for females is expected to be strong and to result in the selection of faster mating or dominant males, and choosy females (Bondurianski 2001; Edward and Chapman 2011).~~ Signals play an essential role in the sexual selection processes, such as male-male contests and mate choice (Searcy and Nowicki 2009; Bro-Jørgensen 2010). In mirid bugs, sex pheromones have been described in a number of species where they are released by females and attract males from ~~the~~ a distance (Wheeler 2001; Zhang and Aldrich 2003; Millar 2005; Byers et al. 2013; Fountain et al. 2014; Yamane and Yasuda 2014). ~~The blend of cuticular hydrocarbons may also play a role in species and sex recognition (Gemeno et al. 2012). However, relatively little is known in mirids about communication modalities other than chemical (Wheeler 2001).~~

Vibrational communication is used by many insect groups, and in particular by Hemiptera (Cokl and Virant-Doberlet 2003; Virant-Doberlet and Cokl 2004). The mechanisms used by insects to produce

vibrational signals are percussion, stridulation, vibration, click mechanisms and air expulsion (Cokl and Virant-Doberlet 2003), while the reception is mediated by mechanoreceptor located at the external surface, like campaniform sensilla, hair sensilla or hairplates, or inside the insect, like scolopidial sensilla or multipolar/multidendritic sensilla (Lakes-Harlan and Strauß 2014). ~~Percussion on the substrate and tremulation of the body are the most common mechanisms used by insects to produce vibrational signals, while the reception is usually mediated by campaniform sensilla and scolopidial organs located in the legs (Cokl and Virant-Doberlet 2003).~~ Homoptera species use vibrational cues almost exclusively ~~vibrational cues~~ for both partner location and courtship (e.g. Mazzoni et al. 2009; de Groot et al. 2012). Heteroptera include some of the best studied species in the field of ~~the~~ bioacoustics, in particular in the families Pentatomidae and Cydnidae (Cokl and Virant-Doberlet 2003; Gogala 2005). Nevertheless, although substrate vibrations are an important signal modality in this group, often in association with sex-pheromones, there are still many families for which mating behavior and vibrational communication are virtually unexplored.

In the family Miridae vibrational signals have been described only in two species: *Lygocoris pabulinus* L. (Groot et al. 1998) and *Lygus rugulipennis* Poppius (Koczor and Čokl 2014). Experiments conducted on these bugs revealed communication in terms of single or successive pulses that, however, in absence of playback experiments, were not associated with~~to~~ any specific function in the context of the courtship behavior. Furthermore, there is not yet evidence of species-specificity of such signals, or if they also occur in intra-gender interactions (i.e. male-male competition).

In the present study we recorded the occurrence of vibrational signals in *M. pygmaeus*. Bioassays with single individuals and intra- and intersex pairs~~,~~ explored the association between the emission of vibrations and specific behavioral contexts. In addition playback experiments were used to elicit behavioral reactions from the tested individuals and thus to assess the role of vibrational signals in absence of other sensorial cues. Finally, to ascertain whether similar signals occurred in a closely related species, and whether they are different for any spectral and/or temporal parameter, we also examined the vibrational signals of the mirid *M. costalis* Fieber.

Materials and Methods

Study species

Insects were reared at the Institut~~o~~ de Recerca i Tecnología Agroalimentàries (IRTA) of Cabrils (Barcelona, Spain) and were shipped as nymphs to the Fondazione Edmund Mach (Trento, Italy). Nymphs were maintained on tobacco plants (*Nicotiana tabacum* L.) provided with frozen moth eggs (*Ephestia kuehniella* Zeller, Lepidoptera: Pyralidae). Newly emerged adults were separated from the colony every 1-2 days so that they were unmated (Castañé et al. 2007) and of known age. To simplify handling, the adults emerged on different days were separated by sex and placed in separate 0.5L plastic containers ~~,~~provided with fresh green bean pods (*Phaseolus vulgaris L.*) and *E. kuehniella* eggs as food, and water sources. Bean pod~~t~~s are a convenient way of keeping batches of insects because they remain fresh for several days without any maintenance (as opposed to a potted plant which would require more space and care) and nymphs can complete development in them (personal observation). The individuals used in the experiments were unmated and between 5 and 15 days of age, counting from the last moult. Climatic conditions in the rearing chambers were 25 ± 1°C, 70 ± 10% RH and a 16:8 (L: D) photoregime with the scotophase starting at 7:00 h local time.

Signal recordings and analysis

A potted tobacco plant was pruned leaving a single leaf ~~which~~ that was trimmed to a surface area of 10-15 cm^2 o~~i~~n each side. A few *E. kuehniella* eggs were deposited on the leaf surface. The plant was placed inside a 30 x 30 x 30 cm clear poly(methyl methacrylate) observation cage. A piece of reflective tape was attached to the top surface of the leaf to reflect the focused beam of a laser vibrometer focus (Ometron VQ-500-D-V, Harpenden, UK) that passed through a hole on the cage. Insects were taken from the rearing box individually with a mouth aspirator and allowed to freely exit it and walk on the leaf surface (Video 1). In recordings with males and females, these were loaded on the plant and allowed to settle for two minutes before loading the males. Recordings took place at the Fondazione Edmund Mach (Trento, Italy) between 9 and 12 h, concurring with the insect scotophase, which is a period of mating activity of *M. pygmaeus* (Gemeno et al. 2007). During recordings there was sufficient light in the test room for the

operators to perform the experiments (approx. 10 lux). ~~*M. pygmaeus* is sexually active during the first half of the day (Gemeno et al. 2007), and apparently was not affected by the ambient light.~~ Different plants were used for each treatment to minimize the potential effect of chemical signals left on the plant by individuals of the other sex (Gemeno et al. 2012).

Recorded vibrations were digitized with a sampling rate of 48 kHz and 16 bit resolution, and stored in a computer using LAN-XI data acquisition hardware (Brüel and Kjær Sound & Vibration A/S, Nærum, Denmark). Behaviour was simultaneously recorded with a camcorder (Panasonic HDCTM700, Hamburg, Germany) equipped with a macro lens (Raynox dcr-25) in order to associate insect movements with the emission of vibrational signals. To describe substrate vibrations generated by each species the following parameters where considered: fundamental and dominant frequency (Hz) of "roar" and "yelp", respectively (see Results), frequency range of "roar" measured within 10 dB from the dominant frequency, signal duration (ms), and inter-pulse interval (IPI, time between two consecutive pulses) of "yelps". In addition to estimating the amplitude of the signals, we also measured the vibratory signal velocity (m/s) of 3 randomly chosen signals of each kind from the *M. pygmaeus* recordings. The velocity was measured at the highest intensity point of the whole signal. Spectral analysis of recorded vibrations was performed with Pulse 14 (Brüel & Kjær Italia SRL, Italy). Recorded vibrations were analyzed with a Fast Fourier Transform (FFT) (type Hann, window length of 512 points and 75 % of overlap). To describe how the frequency ~~modulation~~structure of the "yelp" changes with time we sampled and spectrally characterized 3 distinct parts from each signal: from the onset to ¼ of the length (start), from the onset to ½ of the length (1st half), and from ½ length to the end (2nd half).

Test 1. Characterization of vibrational signals and the effect of social context

M. pygmaeus were recorded individually (single males, single females), in same-sex pairs (male-male, female-female), and in different-sex pairs (male-female) ~~(n = 23 to 28)~~. Recording duration was 1200 seconds, except in male-female pairs where the recording ended when mating occurred. A mixed age group of 20 nymphs of *M. pygmaeus* was recorded in a single occasion. *M. costalis* males were recorded in male-male and male-female pairs to elicit vibrational signals for comparison with those of *M. pygmaeus* ~~(n = 18-50)~~.

Test 2. Playback experiment to assess the role of signals

The ojective of this experiment was to determine if the vibrations alone, produced by males, might influence vibration production and/or walking activity in single males of *M. pygmaeus*. The surface of the tobacco leaf was vibrated with test signals using the conical tip of a 2-cm-long and 4-mm-diameter aluminum rod screwed firmly into the head of a minishaker (Type 4810; Brüel and Kjær, Nærum, Denmark), driven from the computer via Adobe Audition 1.0 (Adobe Software). The tip of the cone was fixed to the leaf with tacky blue wax (Surgident® Periphery Wax Sticks, Heraeus Kulzer, Germany). The playback consisted of two distinct 600-s-long sequences of "roaring" and "yelping" (as main signals emitted by the species; see results of test 1). The "roars" (8-10s long) and "yelps" (8 to 12 individual pulses) used in the playback were recorded during test 1 and were transmitted into the tobacco plant from the basal surface of the leaf (4-5 cm from the distal tip) every 20-30s. Before placing the male on the leaf, we modified the playback signal characteristics using the laser vibrometer and the audio softwares (Pulse 14 and Adobe Audition 1.0) to calibrate them to the values recorded during experiment 1 from living insects.~~The spectral characteristics of playback signals were within the insect´s natural range, and its intensity was calibrated to natural levels with the laser vibrometer~~. As a control, single males were recorded for 600 s in identical conditions but in the absence of playback stimuli.

Statistical analyses

Three randonmly chosen "yelps" and "roars" from each insect recording were averaged and this mean value was used to compare vibrational parameters between the two species using t-tests. To compare the amplitude of “yelp” and “roar” we used the Friedman test with replication, since the data were not normally distributed. In tests 1 and 2 the following variables were analyzed: 1) time of first "roar", "yelp" or contact, 2) duration of each "roar" and of the sum of all "roars", and 3) number of "yelps", "roars" and contacts. The units were as per male, so in the male-male treatment the variables were divided by two (except for number of contacts ~~which~~ that involved both males). Data were very variable and not normally distributed so comparison among treatments was performed with a Kruskal-Wallis test. Pairwise comparisons were performed with a Wilcoxon-Rank test when Kruskal-Wallis was significant, with an alpha level of 0.05 adjusted with Bonferroni correction when more than one comparison was performed.

As control for recordings of different duration (male-female pairs were recorded only until mated) the number and duration of events were divided by the duration of the recording in minutes, and then analyzed. In the playback experiment we counted the number of times that males started a walk, and the total walk duration at the end of the recordings.

Having observed that contacts (only in male-male and male-female pairs) commonly resulted in ~~sound~~ vibration production, we focused on *M. pygmaeus* male-male pairs to assess if contact and ~~sound~~ vibration production were associated. Contact and ~~sound~~ vibration production were scanned for the entire 1200 sec recording period with sampling widths ranging from 1 up to 1200 sec with the goal of determining if the time span between the occurrence of a contact and ~~sound~~ vibration production had an effect on the association between them. The minimum sampling width used was 1-sec because we considered that this duration was a reasonable amount of time to allow the insect to react after a contact. For each sampling width we recorded whether both events (contact and "yelp" or contact and "roar") co-occurred, and the probability of this co-occurrence. The ratio between the probability of "roar" (or "yelp") with contact, and the probability of "roar" (or "yelp") without contact (denominated in here as "relative risk", following standard statistical terminology used in the assessment of the relative difference in the probability of an event under two different conditions, e.g., Agresti 2014), its 95% confidence interval, and the p-value from the Fisher exact test, were also computed for each sampling width.

The analyses were performed with R software (R Core Team 2012). Raw data and R scripts are available for all the tests as Supplementary material.

Results

Test 1. Characterization of vibrational signals and the effect of social context

In both *Macrolophus* species only males produced vibrational signals endowed of specific spectral structure, and thus associable to any behavior (Table 1; Fig. 1), whereas only generic vibrations associated with~~to~~ walking, grooming or egg laying (Video 2) were detected in females (either alone or in pairs). Similarly, a single 30-min recording of a group of 20 *M. pygmaeus* nymphs did not produce any specific vibration (data not shown).

Males produced two types of vibrational signals that we onomatopoeically named "yelp" and "roar". Spectral and temporal features of each signal type are reported in Table 1. "Yelps" are relatively short signals with harmonic structure and regularly decreasing frequency from half length (Fig. 1, Fig. S1). Conversely, "roars" (Fig. 1, Fig. S1) are signals of very variable duration (from 0.02 to 54.75 sec) and with broadband spectral structure. There was no amplitude difference between the two signals: "yelp" 5.56 ± 2.72 µm/s, and "roar" 4.76 ± 3.42 ~~u~~ µm/s (Friedman-Test with replication, df = 1, X^2 = 3,48, P = 0,06). There were significant differences between the two species in the total "yelp" duration, longer in *M. costalis*, and in the "roar" frequency range, which was wider in *M. costalis*, (Table 1).

"Roaring" emission was almost always recorded in association with walking, but males were capable of walking without "roaring" (Video 2). "Yelping" often occurred between walking bouts, when males were stationary. By means of close-up videos we could clearly observe a feeble dorso-ventral body shaking and leg-flexing in association with "yelp" emission, without abdominal substrate contact (Video 3).

Effect of other individuals

"Roaring"

Despite noticeable numeric differences among treatments, the presence of a second individual on the same leaf, either a male or female, did not statistically affect any of the analyzed parameters associated with~~to~~ "roar" emission (Table 2; Fig. 2). However, there was a general trend for increased "roaring"

activity in the presence of a second individual on the leaf. The number of replications in which males emitted "roars" did not significantly differ among group treatments either (23/23, 27/28 and 24/28, for single males, male-male pairs and male-female pairs, respectively; $X^2 = 0.163$; P = 0.922).

"Yelping"

The number of "yelps" per male, and per minute, were significantly higher when males shared the leaf with a second individual, either a male or a female, than when they were ~~single~~ alone (Table 2; Fig. 2). Similarly, the number of individuals that "yelped" at least once was much higher in males paired with another individual, male or female, than in single males (26/28, 24/28 and 6/23, respectively; $X^2 = 6.21$; P = 0.036). The latency of first "yelp" emission was significantly lower for males that were together with another male or with a female than in single males.

"Yelps" occurred in isolation or in tandems of several adjacent "yelps". Occasionally, in male-male pairs, both males "yelped" simultaneously with a rapid succession of interspaced "yelps" resembling call-and-response "yelping duets" (Video 4). Fig. S2~~1~~ shows the frequency distribution of "inter-yelp" intervals for male-male and male-female pairs. The "inter-yelp" interval was slightly shorter in male-male pairs than in male-female pairs (mean ± SEM, 14.02 ± 1.74 and 19.46 ± 3.68 sec, respectively), but there was no significant difference between them (t-test and Mann-Whitney U-test test, both P~~p~~ > 0.05).

Contact and mating

The number of replications where there was at least one physical contact between individuals was similar between male-male and male-female pairs ($X^2 = 0.01$; P = 0.912). The number of contacts per recording was not different between male-male and male-female treatments, but the number of contacts per minute was higher in male-female pairs than in male-male pairs (Table 2). Mating occurred in 54% (15/28) of the male-female pairs, and 87% (13/15) of the matings were preceded by a "yelp" that was emitted immediately before the copulation attempt (Fig.~~ure~~ 2, Videos 5 and 6).

Similarly male-male contacts often resulted in the production of "yelps" (Fig.~~ure~~ 2, Video 4). Table S1 and Fig. 3 show the results for the association between contact and ~~sound~~ vibration production in male-male pairs. The probability of "roaring" was higher when a contact co-occurred during the test

observation period (i.e. sampling width) than when it did not. The association between contact and "roaring" was statistically significant when the sampling width was 3 sec or longer, showing risk ratios higher than 2. Risk ratio peaked at a sampling width of 10 sec (RR = 2.72, Table S1). The association of contact with "yelping" was statistically significant for all sampling widths, and showed the highest risk ratios for 1 and 2 sec sampling widths (RR around 18), followed by a gradual decrease down to 1.45 RR units for the longest sampling width. Overall the co-occurrence of contact and ~~sound~~ vibration production was much higher for "yelps" than for "roars", with probability ratios of "yelp" over "roar" ranging from 7-12 units for sampling widths 1 to 3.

Test 2. Playback experiment to assess the role of the signals

Playback of control, "roar" or "yelp" signals did not change *M. pygmaeus* "roaring" behavior (Table 3). Furthermore, there was almost no "yelping" in this experiment (2 "yelps" in one control male, 7 in a "roar" male and 2 in another "roar" male), so "yelps" were not analyzed. The total amount of emitted "roaring" was about half of that in the first experiment and was not affected by playbacks. However, the total duration of walking was significantly increased (P = 0.02) by "roaring" playback with respect to the control. "Yelp" playback did not affect male behavior. The number of males that "roared" at least once was significantly affected by the playback, so that all the individuals stimulated with the "roar" emitted their own "roar" , whereas in control and "yelp" playbacks only about one third of the individuals "roared" ; $X^2 = 7.55$, P = 0.022).

Discussion

First report in Bricorynae and acoustic parameters

Here we show that two species of the mirid bug *Macrolophus* use substrate-borne vibrational signals for intra-specific communication. This is the first acoustic study in the Bricorynae subfamily, with previous reports on the vibrational communication in the Miridae concerning only the species *Lygocoris pabulinus* L. (Groot et al. 1998) and *Lygus rugulipennis* Poppius (Koczor and Čokl 2014) (both in the subfamily Mirinae). Our study shows a clear difference between the type of signals emitted by these two mirid species and *Macrolophus*. Whilst the two Mirinae bugs were found to emit exclusively one signal type, in the form of pulse trains, the two *Macrolophus* species had a more complex vibrational communication, based on two signals that we onomatopoeically called "yelps" and "roars", and that were affected by the social environment. Furthermore, the mechanism of signal production looks rather different from *L. pabulinus* and *L. rugulipennis*, whose emissions are associated with abdominal vibration and tapping on the substrate, respectively, whereas "yelping" in *Macrolophus* is associated to a slight body shaking (thus resembling a tremulation mechanism, although the use of tymbals or tymbal-like organs in standing males (Video 3) cannot be excluded (Wessel et al. 2014)), and "roaring" is produced by walking males, in the absence of any apparent body vibration.

"Yelp" and "roar" are very different from each other in their acoustic characteristics, the former being slightly over 0.1s in length and with harmonic structure and frequency structure modulation, whereas the latter has broadband structure, intensity modulation and can be produced uninterruptedly for up to many seconds. The narrow-band frequency structure of "yelp" supports the hypothesis of tremulation as a production mechanism of this signal (Elias and Mason 2010). "Yelps" were triggered mostly by encounters with other individuals, and therefore were rarely produced by single males. The "roar" emission, however, was rather conspicuous and spontaneous in both isolated and paired males. Next, we discuss the possible role of these signals in the communication of *Macrolophus*.

Function of "yelping"

In the male-female setting "yelps" were frequent immediately before male copulation attempts, which suggests that this signal has a role in courtship behaviour, for example to increase female motivation to mate. The male´s "yelp" could in this way signal male quality and influence female acceptance (Ringo 1996; Rendall et al. 2009). The relatively high inter-individual variability of the frequency and duration of "yelps", with a coefficient of variation ca. 20% in both *Macrolophus* species, would further support its role in mate choice, because signals with low within- and high between-individual variation may be used to assess the quality of potential partners (Gerhardt 1991). It would be interesting in the future to investigate if "yelps" affect female motivation. Since this signal occurs when the individuals are very close to each other, and in the last moment prior mating, a way to do this is by performing behavioral trials with "silent" males or "deaf" females.

In addition to being produced in a male-female setting, "yelps" were also very common in male-male pairs, in strong association with contacts between the two males. These contacts were frequently followed by what appeared as very brief struggles (although this needs to be confirmed with high-speed video) ensued by both males running away amidst increased "yelping", and occasionally engaging in "yelping duets" lasting up to several seconds (Video 4). This suggests that "yelps", besides being a male-female signal, could also serve as an intra-sexual signal. Male signal exchange could be an indication of dominance, or even territoriality, to monopolize matings, as it would be expected in a highly polygynous and monandrous species, like *Macrolophus* (Gemeno et al. 2007). According to the handicap principle (Zahavi 1975; Smith and Harper 2003), "yelps" could represent an indication of male's quality, thus reducing the necessity of physical contact and fighting between competing individuals. Our "yelp" playback did not trigger the emission of signals on its own, nor did it produce any change in locomotion of test males, which indicates that ~~sound~~ substrate-borne vibration alone is not sufficient to trigger a response in other males, and suggests that other cues (olfactory, gustatory, or visual) may be necessary to start the communication or to elicit a behavioural response in *Macrolophus*.

Function of "roaring"

The strong association between walking and "roaring", and the low specificity and higher variability of its acoustic nature as compared with "yelping", could lead us to think that "roaring" is a mere consequence of locomotion. However, the same individual could "roar" and not "roar" while walking (Video 2), which

strongly suggest that "roar" production is a voluntary behaviour in males. In contrast with "yelping", "roaring" is produced by single males and is weakly associated with male-male contacts, so the role of "roaring" requires a more speculative interpretation than "yelping". *M. pygmaeus* males are highly mobile and take flight easily, whereas females are more sedentary and may use a sex pheromone to attract males, as in other mirids (Zhang and Aldrich 2003; Millar 2005). Therefore under natural conditions it is conceivable that males "advertise" their arrival to a plant (after being lured by the female pheromone) by emitting a long-distance substrate-borne signal, such as "roaring". Because of its relatively large bandwidth a "roar" is likely less susceptible than a "yelp" to be filtered out and dissipate ~~as it~~with distance~~s~~ from the source (Michelsen et al. 1982; Virant-Doberlet et al. 2006; Polajnar et al. 2012). "Roars", in turn, could be intercepted by a second male and a contest may ensue.

Multi-modal communication

While in several Hemiptera species (i.e. Pentatomidae and Auchenorrhyncha) females communicate actively with courting males by emitting their own vibrations in response to male calls (Čokl and Virant-Doberlet 2003; Virant-Doberlet and Čokl 2004; Cocroft and McNett 2005, Mazzoni et al. 2010), *Macrolophus* females have not been observed to produce any vibrational signals, and males must rely on other types of signals to locate them~~females do not produce any vibrational signals, and males must relay in other type of signals to locate them~~. Volatile sex pheromones are prevalent in the family Miridae (Zhang and Aldrich 2003; Millar 2005) and preliminary evidence indicates that *M. pygmaeus* females produce a volatile sex pheromone ~~too~~ (personal observation), so it is possible that male attraction to females in *Macrolophus* is mediated by olfactory signals. Volatile chemical signals do probably play a major role in long-range upwind orientation, but in short distances, such as when a male and female are on the same plant, vision, contact chemical and vibrational signals may also be relevant (Mazzoni et al. 2014). We have observed that male *M. pygmaeus* "yelp" and try to mount stationary females right after contacting them with the antennae, but not before (Video 6), and we have demonstrated that contact between individuals stimulates emission of “yelps”, which suggests that *Macrolophus* relies on contact chemical signals for sexual recognition. Indeed the cuticular hydrocarbon profile of three *Macrolophus* species reveals enough differences between sexes as to allow for mutual recognition (Gemeno et al. 2012). Cuticular hydrocarbon profiles are also very different among the three *Macrolophus* species, so

chemical signals may also be relevant in species recognition, especially if we consider the modest species differences in vibrational parameters between *M. pygmaeus* and *M. costalis* that we have recorded.

We propose a possible ecological scenario for *Macrolophus* vibrational signals in which males, perhaps attracted by a pheromone-releasing female, spontaneously advertise their presence on a plant by means of "roaring", whereas "yelps" are emitted only when the presence of conspecifics (whether male or female) has been detected and assessed by physical contact. Therefore we could envisage the passage from only "roaring" to "roaring" plus "yelping" as a sort of escalation adopted by males to reinforce the basic advertisement signal ("roar") with a more properly qualitative signal ("yelp"), emitted in situations of close vicinity to other individuals, to influence the behaviour of nearby males and females.

Further research is thus warranted to clarify ~~about~~ these issues and to test our hypotheses. A particular focus should be put on mate choice and rivalry systems (for example by means of rivalry bioassays with several males and one female in the same arena and dedicated playback experiments with signal intensity gradients), and on how the different sensorial modalities interact with each other at short range.

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Fig. 1 Acoustic parameters (oscillogram above and spectrogram below) of a pair of "yelps" in *M. pygmaeus* (left) and *M. costalis* (right), and a "roar" produced by *M. pygmaeus* (bottom).

Fig. 2 Temporal dynamics of ~~sound~~ vibration production of *M. pygmaeus* as a function of social context. Males were recorded individually (top graph), in male-male pairs (middle graph) and in male-female pairs (bottom graph). Blank cells: no recording after copulation. ~~Stars~~ Asterisks indicate contact between the two males.

Fig. 3 Global probabilities and probabilities with or without contact of "roaring" (top graph) and "yelping" (middle graph) in *M. pygmaeus* male-male pairs for different sampling widths. Bottom graph: relative risk of ~~sound~~ vibration production, computed as the ratio between probabilities with and without contact, with confidence intervals (gray lines).

table
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Table 1 Spectral and temporal features of roars and yelps of *M. pygmaeus* and *M. costalis* males (mean ± SEM). Statistical comparison between the two species using a t-test (n = 18-50). Statistically significant differences are boldfaced

Sound	Variable[a]	*M. costalis*	*M. pygmaeus*	p-value
	Start	289.94 ± 9.8	289.58 ± 5.18	0.974
	First	299.48 ± 10.81	294.78 ± 4.54	0.692
"Yelp"	Second	251.76 ± 6.67	265.63 ± 4.03	0.085
	FF	299.9 ± 10.74	295.02 ± 5.17	0.685
	Tot	0.16 ± 0.01	0.14 ± 0.00	**0.033**
	Dominant	190.63 ± 12.07	187.70 ± 10.62	0.855
"Roar"	10dB-	100.26 ± 5.86	134.01 ± 6.78	< **0.001**
	10dB+	363.40 ± 12.74	326.60 ± 16.63	0.082

[a]Start: Frequency from onset to ¼ of the length (Hz). First: Frequency from onset to ½ of the length (Hz). Second: Frequency from ½ length to the end (Hz). FF: fundamental frequency (Hz). Tot: total duration (~~m~~s). Dominant: dominant frequency (Hz). 10dB- and 10dB+: lower and upper limit, respectively, of the frequency range 10dB below dominant frequency (Hz).

Table 2 Vibrational parameters as a function of social context. Males of *M. pygmaeus* were recorded for 20 min either singly, paired with another male or paired with a sexually receptive female. Statistically significant differences are boldfaced.

		Male alone		Male-male pair		Male-female pair		Kruskal-Wallis test	
		n	mean (SEM)	n	mean (SEM)	n	mean (SEM)	X^2	P[a]
"Roar"	time of first[b]	23	243.44 (68.09)	24	207.28 (47.81)	27	252.92 (56.45)	0.36	0.834
	number/male	23	11.48 (2.50)	28	19.62 (3.43)	28	16.68 (4.43)	3.75	0.155
	number/male/min	23	0.57 (0.12)	28	0.98 (0.17)	28	1.01 (0.23)	2.21	0.330
	total duration/male	23	30.04 (6.47)	28	68.36 (13.64)	28	53.95 (15.28)	4.25	0.119
	duration each	23	1.50 (0.32)	28	3.42 (0.68)	28	3.46 (0.85)	3.36	0.186
"Yelp"	time of first	6	719.2 (82.63) a	26	401.46 (60.14) b	24	434.51 (68.01) ab	5.99	**0.049**
	number/male	23	0.26 (0.09) b	28	18.1 (3.64) a	28	13.00 (3.08) a	34.52	< **0.001**
	number/male/min	23	0.01 (0) b	28	0.91 (0.18) a	28	1.46 (0.50) a	34.44	< **0.001**
								Wilcoxon rank sum test	
								W	P
Contact	time of first	NA	NA	15	511.69 (80.40)	21	482.53 (73.42)	146	0.721
	number	NA	NA	28	1.29 (0.31)	28	1.82 (0.38)	433	0.224
	number/min	NA	NA	28	0.06 (0.02) b	28	1.14 (0.95) a	535	**0.016**

[a] P-values adjusted with Bonferroni correction

[b] Time is given in seconds

Table 3 Effect of signal playback on "roaring" production and walking of single *M. pygmaeus* males. Statistically significant differences are boldfaced.

		Playback signal							
		Control		"Roar"		"Yelp"		Man-Whitney test (W, P-value)[a]	
Response		n	mean (SEM)	n	mean (SEM)	n	mean (SEM)	Control vs "Roar"	Control vs "Yelp"
"Roar"	time of first[b]	7	139.63 (58.69)	25	111.35 (24.74)	10	188.69 (46.68)	55, 1.00	29, 1.00
	number	20	1.95 (0.86)	25	2.60 (0.5)	30	2.70 (1.17)	201, 0.46	306, 1.00
	total duration	20	6.31 (2.91)	25	19.27 (3.6)	30	8.03 (3.62)	178, 0.16	309, 1.00
Walk	time of first	18	112.33 (28.23)	23	111.74 (17.49)	28	85.68 (18.1)	198, 1.00	261, 1.00
	total duration	20	81.25 (23.2)	24	139.96 (14.99)	30	122.10 (19.03)	150.5, **0.04**	211, 0.16

[a] P-values adjusted with Bonferroni correction

[b] Time is given in seconds

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Fig. 1

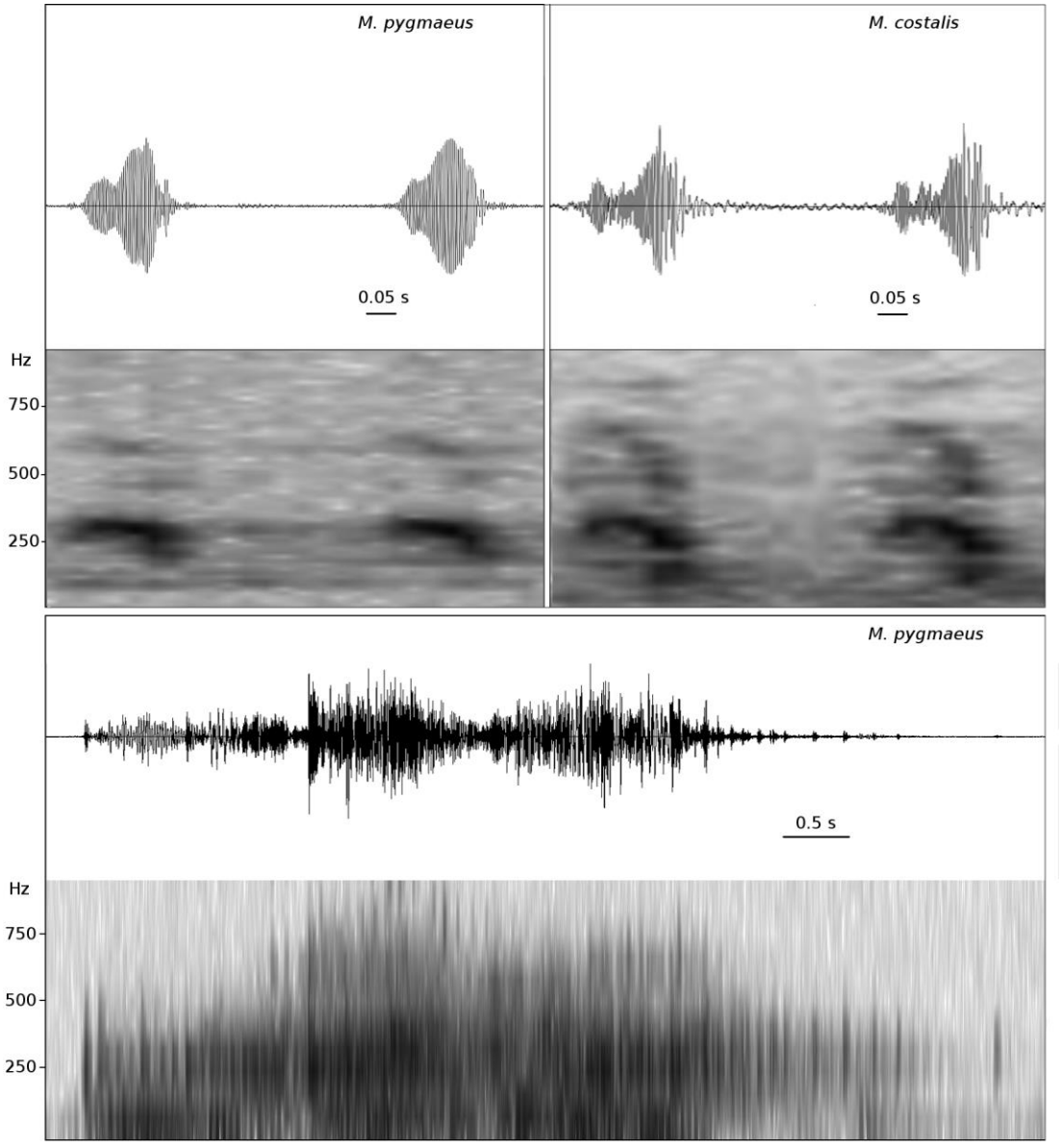


Fig. 2

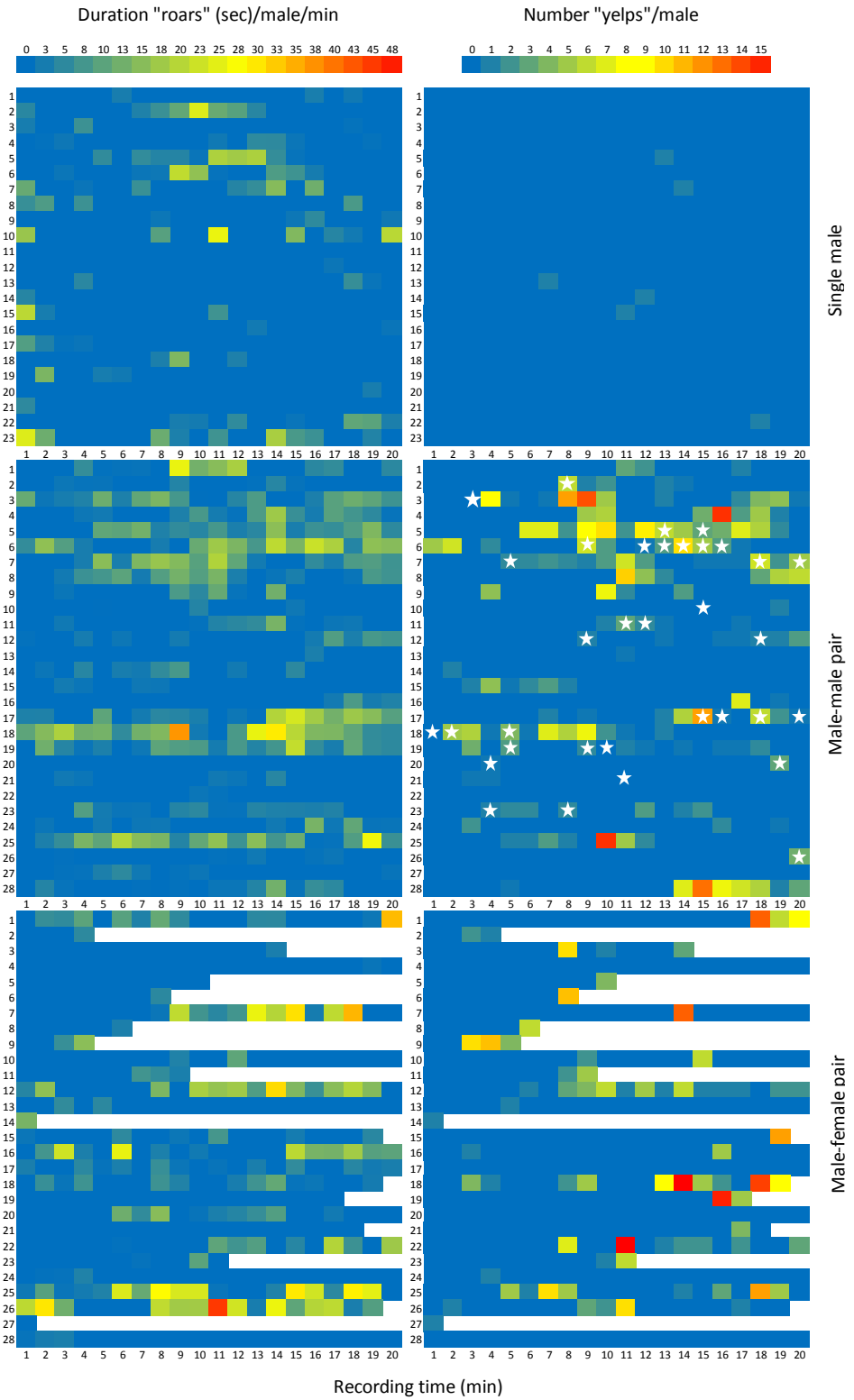
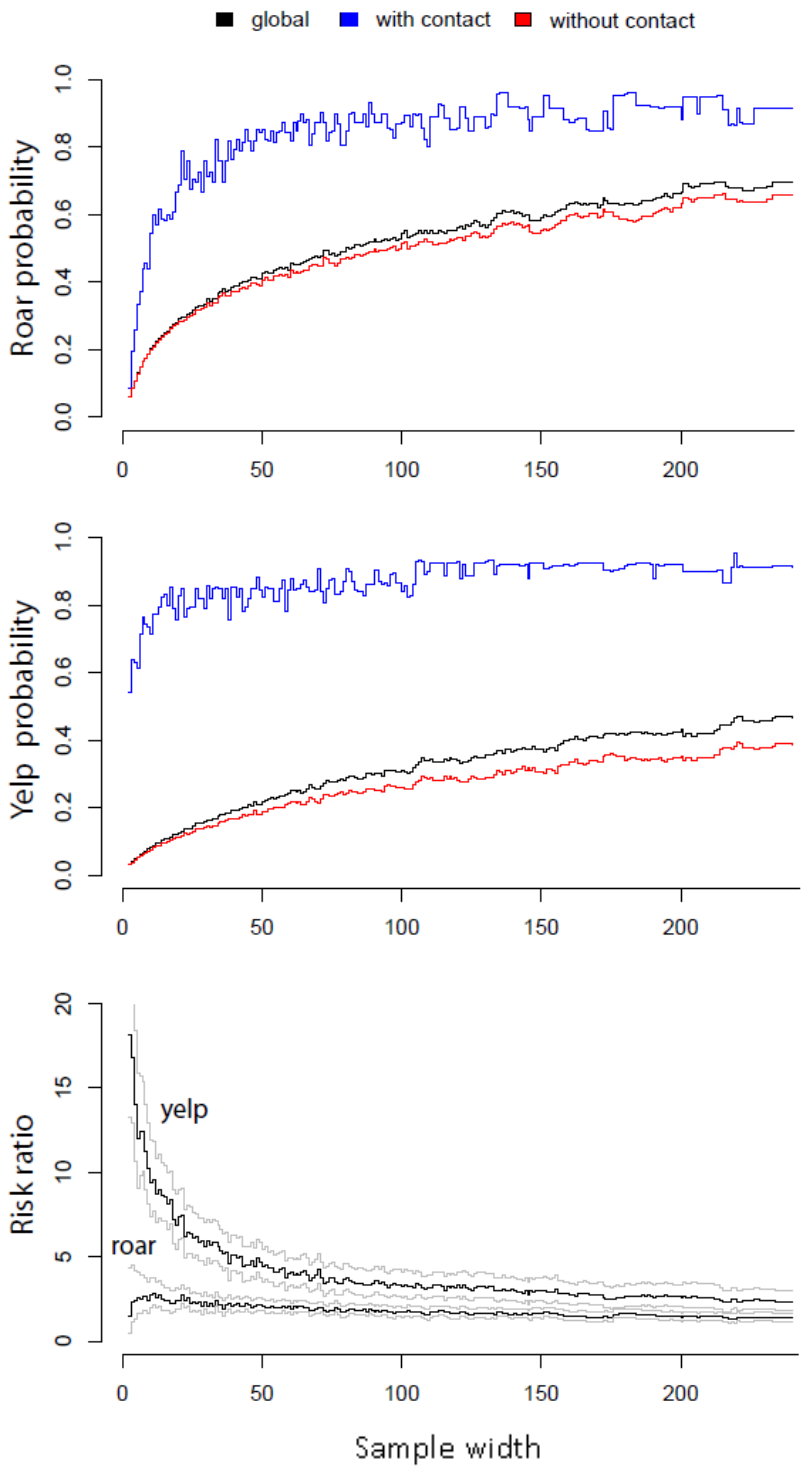

Duration "roars" (sec)/male/min
0 3 5 8 10 13 15 18 20 23 25 28 30 33 35 38 40 43 45 48
Number "yelps"/male
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Single male
Male-male pair
Male-female pair
Recording time (min)

Fig 3


global
with contact
without contact
Roar probability
0.0
0.2
0.4
0.6
0.8
1.0
0
50
100
150
200
Yelp probability
Risk ratio
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